3-29-2017

Hairy root transgene expression analysis of a secretory peroxidase (PvPOX1) from common bean infected by Fusarium wilt

Renfeng Xue
Liaoning Academy of Agricultural Sciences

Xingbo Wu
Tennessee State University

Yingjie Wang
Liaoning Academy of Agricultural Sciences

Yan Zhuang
Liaoning Academy of Agricultural Sciences

Jian Chen
Liaoning Academy of Agricultural Sciences

Recommended Citation

Renfeng Xue, Xingbo Wu, Yingjie Wang, Yan Zhuang, Jian Chen, Jing Wu, Weide Ge, Lanfen Wang, Shumin Wang, Matthew W. Blair, "Hairy root transgene expression analysis of a secretory peroxidase (PvPOX1) from common bean infected by Fusarium wilt", Plant Science, Volume 260, 2017, Pages 1-7, ISSN 0168-9452, https://doi.org/10.1016/j.plantsci.2017.03.011.

Hairy Root Transgene Expression Analysis of a Secretory Peroxidase (*PvPOX1*) from Common Bean Infected by Fusarium Wilt

Renfeng Xue[a,1], Xingbo Wu[b,1], Yingjie Wang[a], Yan Zhuang[a], Jian Chen[a], Jing Wu[c], Weide Ge[a], Lanfen Wang[c], Shumin Wang[c*] , Matthew W. Blair[b*]

[a]Crop Research Institute, Liaoning Academy of Agricultural Sciences, Shenyang, Liaoning 110161, China

[b]Department of Agricultural and Environmental Sciences, Tennessee State University, Nashville, Tennessee 37209, USA

[c]National Key Facility for Crop Gene Resources and Genetic Improvement, Institute of Crop Science, Chinese Academy of Agricultural Sciences, Beijing 100081, China

[1] first two authors contributed equally to this work

*Corresponding authors

E-mail addresses: mblair@tnstate.edu, wangshumin@caas.cn,

Abstract

Plant peroxidases (POXs) are one of the most important redox enzymes in the defense responses. However, the large number of different plant POX genes makes it necessary to carefully confirm the function of each paralogous POX gene in specific tissues and disease interactions. Fusarium wilt is a devastating disease of common bean caused by *Fusarium oxysporum* f. sp. *phaseoli*. In this study, we evaluated a peroxidase gene, *PvPOX1*, from a resistant common bean genotype, CAAS260205 and provided direct evidence for *PvPOX1*'s role in resistance by transforming the resistant allele into a susceptible common bean genotype, BRB130, via hairy root transformation using *Agrobacterium rhizogenes*. Analysis of *PvPOX1* gene over-expressing hairy roots showed it increased resistance to Fusarium wilt both in the roots and the rest of transgenic plants. Meanwhile, the *PvPOX1* expressive level, the peroxidase activity and hydrogen peroxide (H_2O_2) accumulation were also enhanced in the interaction. The result showed that the *PvPOX1* gene played an essential role in Fusarium wilt resistance through the occurrence of reactive oxygen species (ROS) induced hypersensitive response. Therefore, *PvPOX1* expression was proven to be a valuable gene for further analysis which can strengthen host defense response against Fusarium wilt through a ROS activated resistance mechanism.

Keywords: *Agrobacterium rhizogenes*-mediated root transformation, Common bean (*Phaseolus vulgaris* L.), Fusarium wilt (causal agent *Fusarium oxysporum*)

1. Introduction

Fusarium oxysporum spp. are a group of soil-borne fungal pathogens causing Fusarium wilt diseases in many important cash crops worldwide [1]. This economically important group of pathogens attack plants by root infection, and then cause internal blockage of the vascular tissues of the plant stem, and eventually cause plant collapse and death. In common bean, this pathogen species has been classified as *Fusarium oxysporum f. sp. phaseoli* and it has been reported as one of the major diseases that has significant impacts on bean-growing regions around the world but especially in northern China [2].

Type II plant peroxidases (POXs, EC 1.11.1.7) are secreted into the cell wall and are involved in a broad range of growth and development processes [3-5]. Various plant POXs have been shown to be induced by pathogen infection and wounding, suggesting the importance of POX in the defense response to pathogen attack [4]. Plant POXs have been shown to play an essential role in the disease resistance reaction in common beans and other plants through the generation of reactive oxygen species (ROS) in response to fungal or bacterial pathogen infection [6-8]. ROS species have been shown to activate the production of an oxidative burst, the induction of a hypersensitive reaction (HR) and the expression of PR genes involved in plant defense [9, 10]. Peroxidase genes from legumes have been partially studied but few functional analyses of the high number of paralogous sequences have been undertaken using transformation techniques. Legume peroxidases generally belong to large gene families which are made up of a high number of genes in polyploid or large genome species such as soybean (*Glycine max* L. Merr., n=20 haploid complement, 1.1–1.15 Gb genome size) and faba beans (*Vicia faba* L., n= 7 and 4-13 Gb). Common bean which is a true diploid (n=11, and 580 Mb) is thought to have multiple genes in the peroxidase family and expression studies show them to have diverse patterns of RNA transcripts in different tissues [2]. Genome size and gene family number are not always correlated but bigger genomes tend to have larger gene families [11, 12].

The functional analysis of POX genes has depended on either mutagenic or transformation techniques. Transgenic studies of POX genes for overexpression in transgenic plants have been carried out in a large number of species from model plants to horticultural crops but mostly by *Agrobacterium tumefaciens* mediated whole plant transformation [13]. Transposon based knock-out mutants for POX achieved through *A. tumefaciens* plasmid transfer have been studied in rice [14, 15] and Arabidopsis [16]. Meanwhile, transient peroxidase gene analysis has also been achieved by *A. rhizogenes*, which is a soil bacterium closely related to *A. tumefaciens*, but which causes root proliferation and hairy root symptoms [17]. Virus induced gene silencing has been a third method of studying peroxidase effects in plants [18], but is more rarely used.

Although *A. tumefaciens* is the most popular method to perform gene function analysis because it yields genetically-stable whole-plant transgenics, *A. rhizogenes* can be used in some transformation recalcitrant species, to test gene function exclusively in roots. This method shares some of the procedures used with *A. tumefaciens*, but varies in the target tissue, only working in hypocotyl and roots not in stem or leaf tissues. Transformation by *A. rhizogenes* like *A. tumefaciens* uses T-DNA transfer and type IV secretion system to integrate foreign genetic information into the genomic DNA of plant species [19]. However, instead of gall-forming *A. tumefaciens* infection, *A. rhizogenes* infections result in transformed 'hairy' roots which have a chromosomally integrated root-inducing (Ri) plasmid [20]. Hairy root transformation occurs by root sectors creating a "composite or chimeric root system" that is partially transformed but which is useful for evaluating root expressed genes [21].

Legumes are one of the plant groups recalcitrant to *A. tumefaciens* transformation but suitable for *A. rhizogenes* transformation. *A, rhizogenes*-mediated root transformation has been described in many legumes, such as the three forage species, *Lotus corniculatus*, *Trifolium repens*, *Trifolium pratense*, as well as the grain legumes *Vigna*

aconitifolia (moth bean), *Glycine max* (soybean), *Vicia hirsute* (wild faba bean) and in the model species *Lotus japonicus*, *Medicago truncatula* and *Sesbania rostrata* [22-30]. In 2006, Estrada-Navarrete et al. [31] established a fast and reproducible protocol for hairy root transformation of common bean, *P. vulgaris*, and three other species within *Phaseolus*, using *A. rhizogenes* to produce hairy roots on various wild accessions, landraces, and cultivars. The induction of transgenic roots in the genus *Phaseolus* offered a new strategy for functional analysis of root-expressed, endogenous or non-endogenous homologous genes. Based on this efficient method, stable but chimeric transgenic lines can be generated to scale up functional genomics studies in the biology, pathogenesis or microbe interactions of roots.

In this study, we apply hairy root transformation methods to the study of a Fusarium wilt induced peroxidase, which is encoded by a native disease-responsive common bean peroxidase gene, *PvPOX1* [2]. The study by Xue et al. [32] demonstrated that the H_2O_2 burst struck at the time of *PvPOX1* gene expression along with the HR response and accompanying programmed cell death, suggesting the gene's important role in the resistant mechanism to Fusarium wilt in common bean. However, full functional analysis of the PvPOX1 protein was missing as expression studies were correlative rather than giving causative results as can be obtained in transformation analysis. Therefore, we used *A. rhizogenes* and hairy root transformation to analyze the role of the PvPOX1 gene product in conferring Fusarium wilt resistance into a *Fusarium* susceptible common bean genotype, BRB130 compared to the original source or resistance, CAAS260205, which is a novel germplasm entry from China. In addition to evaluating the disease response phenotype of the control and transformed plants of BRB130 compared to CAAS260205, we also studied *PvPOX1* expression, POX activity and H_2O_2 accumulation under the pathogen infection in both *PvPOX1* transgenic susceptible and non-transgenic, resistant and susceptible control plants.

2. Materials and Methods

2.1. Plant materials and PvPOX1 source

Common bean germplasm was provided by the Institute of Crop Sciences (ICS), Chinese Academy of Agricultural Sciences (CAAS) in Beijing, China. They included two different genotypes: CAAS260205 (Fusarium wilt resistant) and BRB130 (Fusarium wilt susceptible) [33]. CAAS260205 has small black seed and is of the Mesoamerican (or Middle American) genepool and was used in extensive Fusarium wilt testing by Xue et al. [2]. BRB130 is a breeding line of the Andean genepool with white color. It is resistant to Bean Common Mosaic Necrosis Virus (BCMNV) and Black Root Symptoms and was confirmed by marker analysis to have the dominant *I* gene (unpublished data, M. Blair) but is susceptible to Fusarium wilt through tests done here and by Xue et al. [2, 32]. The *PvPOX1* gene (Genbank accession no. JQ627838) source was as described in these previous papers.

2.2 Fungal isolate, pathogen inoculation and disease scoring

An aggressive *F. oxysporum* f. sp. *phaseoli* (*Fop*) isolate, FOP-DM01 was used for the experiments as further described by Xue et al. [33]. The fungal isolate was grown in darkness on petri dishes with potato dextrose agar (PDA) medium at a constant 25°C. Prior to inoculation, the bean seeds were sown in 15-cm diameter pots filled with sterile vermiculite and clay at a volumetric ratio of 3:1. The seedlings were allowed to grow in natural greenhouse conditions at 22 to 28°C and 35 to 40% humidity up to 10 days (d) of age. At this point non-inoculated control plants were separately labeled from plants to be infected with the pathogen so as to have both gene and biochemical reaction from infected and uninfected plants The 10 d old seedlings were grown for an addition day to be used for infection by Fop at the fully expanded unifoliolate leaf stage. Plants were infected by the isolate FOP-DM01 using a previously described method [33].

The non-inoculated, control plants were transferred to non-inoculated mixture and provided the same growth conditions along with adequate watering after transplanting. The three plants for each treatment and each time point were grown in randomized block design and fertilized every week. The plants were grown in a greenhouse maintained at approximately 22–28°C. All treatments were applied in the same greenhouse with daylight plus 14 h of supplemental lighting. The plants with and without transgenic hairy root transformation as described below were inoculated with the same methods. The disease assessments were performed using a one to nine disease severity scale described in Schoonhoven et al. [34] with individual plant assessments in three repetitions. *PvPOX1* gene expression, POX and H_2O_2 analysis were performed as described in Xue et al. [2].

2.3. Transgenic hairy root cultivation and identification

The p35SGFPGUS+ binary vector (Genbank accession number：EF546437) provided by Vickers et al. [35] was used for construction of a new *PvPOX1* gene overexpression construct. The open reading frame (ORF) fragment of *PvPOX1* amplified as described by Xue et al. [2] was digested by *Xba*I/*Sac*I and was subsequently ligated into a mini-prep of the p35SGFPGUS+ plasmid that was similarly digested with the same enzymes. This recombinant binary vector had a 35S promoter constructed to drive the *PvPOX1* gene, which was followed by the NOS terminator. The plasmid DNA mini-preps were carried out with Qiagen kit both for cloning and transfection steps. The new construct was electroporated into the *E. Coli* strain DH5α for storage and multiplication. DNA was isolated from that strain for transformation purposes. The transformation procedure mediated by cucumopine-type *Agrobacterium rhizogenes* strain K599 followed the method of Estrada-Navarrete et al. [31] with vectors kindly provided by F. Sanchez, director of that lab. A plasmid was generated with an empty vector (EV treatment) to compare to transformation with the *PvPOX1* gene (PvPOX1 treatment) as a control for gene expression along with non-transformed, no-vector (NT) treatment as control for transformation.

In the case of the PvPOX1 transgenic plants, the transformation strain was grown on LB media with 50ng/ml concentration of Kanamycin. Meanwhile, fresh cultures of *A. rhizogenes* K599 strains with empty overexpression vector or without a binary vector were grown in LB medium without antibiotics for EV and NT plant generation of the susceptible genotype BRB130. Therefore, in both cases the controls had no *PvPOX1* gene in the hairy root system inoculation treatment and the expected phenotype was susceptible. The primers PXD1-Forward (5'-CACTCTAAGCTCAGCTCAACTCAC) and PXD2-Reverse (5'-TTGGGGTCCTTATCAACAGC) were designed to confirm presence of the *PvPOX1* gene allele and for positive transgenic root identification. PCR amplification used a denaturation temperature of 92 °C, an annealing temperature of 58 °C and an extension temperature of 72 °C in a standard thermocycle of 1 minute of denaturation, 1 minute of annealing and 2 minutes of extension, repeated for 35 cycles. *Taq* polymerase from TaKaRa and a standard PCR machine were used to detect the bands on 1% agarose gels in 1 X TAE buffer.

2.4. PvPOX1 gene expression, POX and H_2O_2 analysis

Gene expression analysis for *PvPOX1* was evaluated with quantitative real-time PCR analysis (qRT-PCR) in the roots and stems tissues of common bean from various treatments described above. Total RNA for the RT-PCR step was extracted from 500 mg of the bean root frozen in liquid N_2 using TRNzol-A+ Reagent (TianGen, Beijing, China) according to the manufacturer's instructions. The total RNA samples were treated using DNase I (TaKaRa, Dalian, China) to eliminate residual genomic DNA. Then, 20 ng of total RNA was used for first-strand cDNA synthesis, followed by second-strand synthesis, carried out with the Universal RiboClone cDNA Synthesis System (Promega, Madison, WI, USA) following the manufacturer's instructions. This cDNA product was used for qPCR analysis. The primers of *PvPOX1*-specific PCR along with the reaction mixture and PCR program were as described in Xue et al. [32]. As in that previous study, a *P. vulgaris* actin gene was used as an internal control to standardize gene expression differences.

Peroxidase activity assays were carried out with a previously published method [36]. One unit of enzyme activity was defined as the amount of enzyme converting 1 μmol of substrate to product in 1 min. Total H_2O_2 content was measured by the method of Sagisaka [37] using a standard curve based on plant H_2O_2 . The test tubes contained the extract equivalent of 0.05 g dry weight sample in a volume of 1.60 ml. To this solution was added 0.4 ml of 50% trichloroacetic acid, 0.4 ml of 10 mM ferrous ammonium sulfate, and color developed by the addition of 0.2 ml of 2.5 M potassium thiocyanate was read at 480 nm.

3. Results

3.1. PvPOX1 gene expression

Real-time PCR analysis was conducted to examine PvPOX1 gene expression level in roots and stems of CAAS260205 and BRB130 infected by *F. oxysporum* f. sp. *phaseoli* isolate FOP-DM01. The results indicated that the transcription of *PvPOX1* was strongly and rapidly induced in roots and stems by Fusarium infection. mRNA levels of the *PvPOX1* gene in the roots of CAAS260205 increased from 0 to 4 h, then declined at 8 h and reached the peak of 5.3-fold over control at 24 h. From 48 to 120 h, transcription decreased to 2.1-fold and then steadily increased to 3.4-fold (Fig. 1A). In the stems of CAAS260205, the expression degree of *PvPOX1* gene decreased from 0 to 12 h, and then dramatically increased until 120 h, the maximum level of more than 100-fold was reached at 96 h (Fig. 1B). There was no significant gene expression changes observed both in roots and stems of genotype BRB130 (Fig. 1).

3.2. Peroxidase activity assay

Total peroxidase activity was determined in roots and stems induced by Fop-DM01 isolate during the time points selected. As mentioned by Xue et al. [32], *PvPOX1* expression generally increased during the HR establishment and, for the most part, peroxidase activity paralleled the accumulation of PvPOX1 mRNAs. As shown in the Figure 2A, POX activity in roots of CAAS260205 increased from 8.9 to 14.7 μmol/min/mg fresh weight after 24 h post inoculation, and a late peak was reached at 96 h, when the HR was visible at the infected sites after 72 h post inoculation. Meanwhile, POX activity in stems of CAAS260205 increased after 24 h post inoculation and also reached the maximum level of 15.3 μmol/min/mg fresh weight at 96 h (Fig. 2B). However, no significant changes were detected in POX activity over time in the bean roots and stems of un-inoculated CAAS260205 and both inoculated and non-inoculated BRB130 control.

3.3. H_2O_2 accumulation

In this study, we monitored the H_2O_2 accumulation levels in infected roots and stems at all time points after inoculation. In the un-inoculated plants, no significant changes were detected. A rapid oxidative burst occurred in infected roots and stems of CAAS260205 relatively early in post inoculation, reaching an approximately 3.8-fold difference compared to control plants at 4 h post inoculation in the infected roots (Fig. 3A) but subsiding at 8h (Fig. 3B). Apart from the first oxidative burst of the HR reaction in the infected tissues, a striking second oxidative burst occurred, in the 72-120 h time interval and in both roots and stems. The maximum accumulations of H_2O_2 were 5.7-fold in roots and 1.8-fold in stems at 120 h after infection compared with un-inoculated CAAS260205 control and inoculated and non-inoculated BRB130 plants which did not show any significant change in the overall time course (Fig. 3). Lack of differences in the BRB130 genotype is typical of Fusarium wilt susceptible genotypes, which is why it was the genotype chosen for transgenesis experiments.

3.4. A. rhizogenes-mediated root transformation

Induction of *A. rhizogenes*-mediated root transformation in this experiment was referenced from the Phaseolus spp. transformation protocol using *A. rhizogenes* strain K599 [31, 38] Hairy roots were approximately 3-5 cm in length (Fig. S1A) after 14 d post induction. Then plants were transferred to a new pot with new sterile vermiculite supplemented with B&D solution. Each pot contained 1-3 plants and cover hairy roots up to 1 cm with sterile vermiculate. Keep plants in a growth chamber (16 h light/8 h dark at 25 – 28℃) until hairy roots emerge (Fig. S1B). After 21 d post transferring, the primary root was cut at the stem attachment site, 2 cm below the development of hairy roots (Fig. S1C), and then the hairy roots of plants without the primary root were used for the identification of positive transgenics (Fig. S1D) and further resistance assessment against FOP-DM01 isolate.

Resistance assessment of transgenic plants

The results indicated the phenotypic differences between control and *PvPOX1* transgenic plants infected by FOP-DM01 isolate (Fig. 4A). Infected hairy roots of both non-transformed control plants (NT) and control plants with empty overexpression vector (EV) showed the typical reddening and yellowing color caused by Fusarium wilt pathogen at 14 days post inoculation, and leaf chlorosis, stem browningalso appeared as the typical visible symptoms, however, the *PvPOX1* transgenic plants only showed a slight stunting and reddening in the *PvPOX1* transgenic hairy roots in comparison to the both control plants at 14 days post inoculation. This indicated *PvPOX1* overexpression enhanced the resistance of the hairy roots, even the overall bean seedling to *F. oxysporum* drastically and prevented fungal colonization in the remaining parts of the plant. In this study we showed that root reddening, vascular browning, wilting, and leaf yellowing were significantly greater ($P<0.05$) in both control plants than in *PvPOX1*-transgenic plants (Fig. 4B). Therefore, we demonstrated for the first time that *PvPOX1* gene overexpression in common bean can enhance the resistance of hairy roots and overall plants against the Fusarium wilt pathogen FOP-DM01 isolate.

In the analysis of *PvPOX1* gene overexpression plants, we found that *PvPOX1* expression degree was strongly induced in hairy roots challenged by *F. oxysporum* f. sp. *phaseoli* isolate FOP-DM01 (Fig. 5A). It demonstrated that relative expression level of the *PvPOX1* gene in *PvPOX1* transgenic plants increased dramatically at 7 d post inoculation and reached a 3.4-fold of control level, and then decreased to 2.7-fold at 14 d after infection. However, the two control plants (NT and EV) did not change significantly and even declined at 14 d post inoculation. In general, peroxidase activity paralleled the accumulation of *PvPOX1* mRNAs (Fig. 5B). The POX activity of *PvPOX1* transgenic hairy roots increased from 10.4 to 15.4 μmol/min/mg fresh weight at 0-7 d after pathogen infection, and decreased to 9.2 μmol/min/mg fresh weight at 14 d post inoculation, however, the POX activity of both control hairy roots

decreased gradually. The H_2O_2 levels in hairy roots infected with Fusarium pathogen were also analyzed in this study (Fig. 5C). The significant reduction occurred in control bean hairy roots after inoculation. In comparison, the levels of H_2O_2 observed in *PvPOX1* transgenic plants at 7 d after infection reached approximately 0.37 ng/mg fresh weight, and 0.24 ng/mg fresh weight at 14 d post inoculation. The H_2O_2 levels significantly increased in comparison with that in control plants. These results indicated that the increase in expression level of the *PvPOX1* gene, the total peroxidase activity and the levels of H_2O_2 were all correlated.

4. Discussion

The first major achievement of this study was to use hairy root transformation to test the functional role of an apoplastically-secreted, peroxidase gene (*PvPOX1*) isolated by Xue et al. [32]. The *PvPOX1* gene was found from a novel resistant landraces called CAAS260205. The gene was originally identified in a cDNA library of common bean roots challenged with the Fusarium wilt pathogen by isolate FOP-DM01 of *F. oxysporum* f. sp. *phaseoli* , and its expression level was upregulated significantly in the resistant genotype under pathogen attack [38], so was considered a good candidate for the functional and transgenic analysis carried out here.

Another key achievement of this study was to apply the hairy root transformation system of Estrada-Navarrete [38] to the study of plant stresses. While we evaluated biotics stress resistance, other authors have evaluated peroxidases in roots using hairy root transformation but for abiotic stresses. For example Oller et al. [41] established transgenic tomato hairy root clones which overexpress a basic peroxidase gene *tpx1*, mediated by *Agrobacterium rhizogenes* for phenol removal in soils. Cao et al. [42] showed that *TaNHX2* gene could enhance salt tolerance of soybean, and *A. rhizogenes*-mediated transformation system could be used as a complementary tool of *A. tumefaciens*-mediated transformation to rapidly investigate candidate gene function in soybean. However, *A. rhizogenes*-mediated transformation system in common bean has been established, but not applied in root candidate gene function identification.

Fusarium wilt, caused by *Fusarium oxysporum* Schlecht. f. sp. *phaseoli* Kendrick and Snyder, is a major disease of the common bean (*Phaseolus vulgaris* L.). The plants are invaded by the pathogen through the roots where the xylem is colonized, causing wilting, vascular discoloration, chlorosis, dwarfism, and premature plant death [39]. Plant peroxidases are present in all land plants and their remarkable catalytic versatility allows them to be involved in a broad range of physiological and developmental processes throughout the plant life cycle [4].

Here, we constructed an overexpression plasmid containing the common bean peroxidase most likely involved in Fusarium wilt resistance, *PvPOX1*, and established transgenic hairy roots for the functional analysis of the gene in common bean following the *A. rhizogenes*-mediated transformation method of Estrada-Navarrete et al. [38]. The result indicated *PvPOX1* gene overexpression enhanced the defense response in hairy roots, and even overall common bean plants to Fusarium wilt pathogen, the total peroxidase activity and H_2O_2 levels in hairy roots were in accompany with the increasing expression of the *PvPOX1* gene. Our hypothesis is that the increase of *PvPOX1* gene caused the enhancement of total peroxidase activity and H_2O_2 levels in the *PvPOX1* transformed hairy roots, further causing stronger HR occurrence and defense activation towards pathogen attack as was seen by [36].

In terms of *PvPOX1* gene expression pattern, the total peroxidase activity and H_2O_2 levels in the roots and stems of the resistant genotype CAAS260205 infected by *F. oxysporum* f. sp. *phaseoli* was higher than for BRB130, until this susceptible genotype was transformed and achieved a similar level. The data indicated, therefore, that the *PvPOX1* gene played a key role in the ROS formation causing a defense response in common beans. It also demonstrated the *PvPOX1* gene was upregulated to induce the increase of total peroxidase activity and H_2O_2 levels in the *PvPOX1* overexpression analysis. Therefore, *PvPOX1* expression enhancement strengthened the host defense response, and activated resistant mechanism mediated by ROS against Fusarium wilt pathogen.

This peroxidase activity and biphasic accumulation of H_2O_2 during the plant oxidative burst has been found in other plant–pathogen interactions [10]. For example, H_2O_2 was produced in a first oxidative burst between 0 and 2 h and in a second more intense oxidative burst between 8 and 10 h after inoculation in resistant tobacco cells inoculated with an aggressive *P. nicotianae* [40]. In our study, when transcription of the *PvPOX1* gene increased, peroxidase activity increased correspondingly, while the

H_2O_2 accumulation increased in a parallel and progressive two-step manner. The gene expression data from both roots and stems when coupled with the pattern of variation found for the peroxidase activity and the H_2O_2 levels implies the *PvPOX1* gene plays an important role in plant defense through ROS formation. Therefore, we suggest that the striking increase of H_2O_2 caused by *PvPOX1* expression strong enhancement was the key regulator which can be associated with the HR response.

Further research is needed to illuminate the full molecular mechanisms controlling resistance to Fusarium wilt pathogen as well as additional molecular components that mediate the interaction between plant and pathogen. Part of these upcoming studies could evaluate the cellular localization of H_2O_2 burst using fluorescence probes in microscopy work for determining if the response is limited to one subcellular compartment or another. Given our main goal to evaluate the resistance provided by the *PvPOX1* gene to the fusarium wilt pathogen it would also be advisable to attempt biolistics or *A. tumefaciens*-mediated whole-plant transformation which could also be used for the verification of other candidate genes in common beans [43]. We can conclude, however, that *A. rhizogenes* has a tremendous role to play in the functional analysis of root specific genes and therefore possesses enormous potential as a molecular tool in the breeding of Fusarium wilt resistant bean varieties and perhaps more broadly in the development of biotic-stress tolerant legume genotypes.

Acknowledgements

We thank Dr. Sánchez F. (Departamento de Biología Molecular de Plantas, Instituto de Biotecnología, Universidad Nacional Autónoma de México) for supplying the binary vector and Dr. W. Messier (Evolutionary Genomics, Boulder Colorado USA) for helpful advice on promoter and gene constructs. The authors acknowledge funding provided by Liaoning Doctor Startup Foundation (201501113), the Natural Science Foundation of China (NSFC) (31401447), Modern Agro-industry Technology Research System (CARS-09-8Z), New Varieties Breeding and Cultivation Techniques of Sorghum and Characteristic Grains (201401651-3), Evolutionary Genomics (EG Crop Science Inc.) and the Bill and Melinda Gates Foundation and the Evans Allen Fund for Agricultural Research at Tennessee State University.

References

[1] R.A. Buruchara, L. Camacho, Common bean reaction to *Fusarium oxysporum* f. sp. *phaseoli*, the cause of severe vascular wilt in Central Africa, Journal of Phytopathology 148 (2000) 39-45.

[2] R. Xue, J. Wu, Z. Zhu, L. Wang, X. Wang, S. Wang, M.W. Blair, Differentially expressed genes in resistant and susceptible common bean (*Phaseolus vulgaris* L.) genotypes in response to *Fusarium oxysporum* f. sp. *phaseoli*, PloS One 10 (2015) e0127698.

[3] N. Bakalovic, F. Passardi, V. Ioannidis, C. Cosio, C. Penel, L. Falquet, C. Dunand, PeroxiBase: a class III plant peroxidase database, Phytochemistry 67 (2006) 534-539.

[4] F. Passardi, C. Cosio, C. Penel, C. Dunand, Peroxidases have more functions than a Swiss army knife, Plant cell Rep. 24 (2005) 255-265.

[5] L. Almagro, L.G. Ros, S. Belchi-Navarro, R. Bru, A.R. Barceló, M. Pedreno, Class III peroxidases in plant defense reactions, J. Exp. Bot. 60 (2009) 377-390.

[6] R. Mittler, S. Vanderauwera, M. Gollery, F. Van Breusegem, Reactive oxygen gene network of plants, Trends Plant Sci. 9 (2004) 490-498.

[7] K.A. Blee, S.C. Jupe, G. Richard, A. Zimmerlin, D.R. Davies, G.P. Bolwell, Molecular identification and expression of the peroxidase responsible for the oxidative burst in French bean (*Phaseolus vulgaris* L.) and related members of the gene family, Plant Mol Biol. 47 (2001) 607-620.

[8] G.P. Bolwell, L.V. Bindschedler, K.A. Blee, V.S. Butt, D.R. Davies, S.L. Gardner, C. Gerrish, F. Minibayeva, The apoplastic oxidative burst in response to biotic stress in plants: a three-component system, J. Exp. Bot. 53 (2002) 1367-1376.

[9] J.D. Jones, J.L. Dangl, The plant immune system, Nature 444 (2006) 323-329.

[10] C. Lamb, R.A. Dixon, The oxidative burst in plant disease resistance, Annu. Rev. Plant Biol. 48 (1997) 251-275.

[11] D.E. Soltis, P.S. Soltis, M.D. Bennett, I.J. Leitch, Evolution of genome size in the angiosperms, Am. J. Bot. 90 (2003) 1596-1603.

[12] J. Schmutz, S.B. Cannon, J. Schlueter, J. Ma, T. Mitros, W. Nelson, D.L. Hyten, Q. Song, J.J. Thelen, J. Cheng, Genome sequence of the palaeopolyploid soybean, Nature 463 (2010) 178-183.

[13] K. Yoshida, P. Kaothien, T. Matsui, A. Kawaoka, A. Shinmyo, Molecular biology and application of plant peroxidase genes, Appl. Microbiol. Biot. 60 (2003) 665-670.

[14] Z. Zhang, Q. Zhang, J. Wu, X. Zheng, S. Zheng, X. Sun, Q. Qiu, T. Lu, Gene knockout study reveals that cytosolic ascorbate peroxidase 2 (*OsAPX2*) plays a critical role in growth and reproduction in rice under drought, salt and cold stresses, PloS One 8 (2013) e57472.

[15] A. Bonifacio, M.O. Martins, C.W. Ribeiro, A.V. Fontenele, F.E. Carvalho, M. MARGIS-PINHEIRO, J.A. Silveira, Role of peroxidases in the compensation of cytosolic ascorbate peroxidase knockdown in rice plants under abiotic stress, Plant Cell Environ. 34 (2011) 1705-1722.

[16] G. Miller, N. Suzuki, L. Rizhsky, A. Hegie, S. Koussevitzky, R. Mittler, Double mutants deficient in cytosolic and thylakoid ascorbate peroxidase reveal a complex mode of interaction between reactive oxygen species, plant development, and response to abiotic stresses, Plant Physiol. 144 (2007) 1777-1785.

[17] A. Riker, W. Banfield, W. Wright, G. Keitt, H.E. SAGEN, Studies on infectious hairy root of nursery apple trees, J. Agr. Res. 41 (1930) 507-540.

[18] J.-E. Wang, K.-K. Liu, D.-W. Li, Y.-L. Zhang, Q. Zhao, Y.-M. He, Z.-H. Gong, A novel peroxidase *CanPOD* gene of pepper is involved in defense responses to *Phytophtora capsici* infection as well as abiotic stress tolerance, Int. J. Mol. Sci. 14 (2013) 3158-3177.

[19] S.C. Winans, D.L. Burns, P.J. Christie, Adaptation of a conjugal transfer system for the export of pathogenic macromolecules, Trends Microbiol. 4 (1996) 64-68.

[20] M.-D. Chilton, D.A. Tepfer, A. Petit, C. David, F. Casse-Delbart, J. Tempé, *Agrobacterium rhizogenes* inserts T-DNA into the genomes of the host plant root cells, Nature 295 (1982) 432-434.

[21] R. Collier, B. Fuchs, N. Walter, W. Kevin Lutke, C.G. Taylor, *Ex vitro* composite

plants: an inexpensive, rapid method for root biology, Plant J. 43 (2005) 449-457.

[22] A. Petit, J. Stougaard, A. Kühle, K.A. Marcker, J. Tempé, Transformation and regeneration of the legume *Lotus corniculatus*: a system for molecular studies of symbiotic nitrogen fixation, Mol. Gen. Genet. 207 (1987) 245-250.

[23] C.L. Diaz, L.S. Melchers, P.J. Hooykaas, B.J. Lugtenberg, J.W. Kijne, Root lectin as a determinant of host-plant specificity in the Rhizobium-legume symbiosis, Nature 338 (1989) 579-581.

[24] C.L. Díaz, H.P. Spaink, J.W. Kijne, Heterologous rhizobial lipochitin oligosaccharides and chitin oligomers induce cortical cell divisions in red clover roots, transformed with the pea lectin gene, Mol. Plant-Microbe In. 13 (2000) 268-276.

[25] N.G. Lee, B. Stein, H. Suzuki, D.P.S. Verma, Expression of antisense nodulin-35 RNA in *Vigna aconitifolia* transgenic root nodules retards peroxisome development and affects nitrogen availability to the plant, Plant J. 3 (1993) 599-606.

[26] C.I. Cheon, N.G. Lee, A. Siddique, A.K. Bal, D. Verma, Roles of plant homologs of Rab1p and Rab7p in the biogenesis of the peribacteroid membrane, a subcellular compartment formed *de novo* during root nodule symbiosis, EMBO J. 12 (1993) 4125.

[27] J. Stiller, L. Martirani, S. Tuppale, R.-J. Chian, M. Chiurazzi, P.M. Gresshoff, High frequency transformation and regeneration of transgenic plants in the model legume *Lotus japonicus*, J. Exp. Bot. 48 (1997) 1357-1365.

[28] A. Boisson-Dernier, M. Chabaud, F. Garcia, G. Bécard, C. Rosenberg, D.G. Barker, *Agrobacterium rhizogenes*-transformed roots of *Medicago truncatula* for the study of nitrogen-fixing and endomycorrhizal symbiotic associations, Mol. Plant-Microbe In. 14 (2001) 695-700.

[29] W. Van de Velde, J. Mergeay, M. Holsters, S. Goormachtig, *Agrobacterium rhizogenes*-mediated transformation of *Sesbania rostrata*, Plant Sci. 165 (2003) 1281-1288.

[30] H.J. Quandt, A. Pühler, I. Broer, Transgenic root nodules of *Vicia hirsuta*: a fast and efficient system for the study of gene expression in indeterminate-type nodules,

515 Mol. Plant-Microbe In. 6 (1993) 699-706.
516 [31] G. Estrada-Navarrete, X. Alvarado-Affantranger, J.-E. Olivares, C. Díaz-Camino,
517 O. Santana, E. Murillo, G. Guillén, N. Sánchez-Guevara, J. Acosta, C. Quinto,
518 *Agrobacterium rhizogenes* transformation of the *Phaseolus* spp.: a tool for
519 functional genomics, Mol. Plant-Microbe In. 19 (2006) 1385-1393.
520 [32] R.F. Xue, J. Wu, M.L. Chen, Z.D. Zhu, L.F. Wang, X.M. Wang, M.W. Blair, S.M.
521 Wang, Cloning and characterization of a novel secretory root-expressed peroxidase
522 gene from common bean (*Phaseolus vulgaris* L.) infected with *Fusarium*
523 *oxysporum* f. sp. *phaseoli*, Mol. Breeding 34 (2014) 855-870.
524 [33] R. Xue, Z. Zhu, Y. Huang, X. Wang, L. Wang, S. Wang, Quantification of
525 *Fusarium oxysporum* f. sp. *phaseoli* detected by real-time quantitative PCR in
526 different common beans cultivars, Acta. Agron. Sin. 38 (2012) 791-799.
527 [34] A.V. Schoonhoven, M.A. Pastor-Corrales, Standard system for the evaluation of
528 bean germplasm, International Center for Tropical Agriculture (CIAT), Cali,
529 Columbia, 1987.
530 [35] C.E. Vickers, P.M. Schenk, D. Li, P.M. Mullineaux, P.M. Gresshoff,
531 pGFPGUSPlus, a new binary vector for gene expression studies and optimizing
532 transformation systems in plants, Biotechnol. Lett. 29 (2007) 1793-1796.
533 [36] H.M. Do, J.K. Hong, H.W. Jung, S.H. Kim, J.H. Ham, B.K. Hwang, Expression
534 of peroxidase-like genes, H_2O_2 production, and peroxidase activity during the
535 hypersensitive response to *Xanthomonas campestris* pv. *vesicatoria* in *Capsicum*
536 *annuum*, Mol. Plant-Microbe In. 16 (2003) 196-205.
537 [37] S. Sagisaka, The occurrence of peroxide in a perennial plant, *Populus gelrica*,
538 Plant Physiol 57 (1976) 308-309.
539 [38] G. Estrada-Navarrete, X. Alvarado-Affantranger, J.-E. Olivares, G. Guillén, C.
540 Díaz-Camino, F. Campos, C. Quinto, P.M. Gresshoff, F. Sanchez, Fast, efficient
541 and reproducible genetic transformation of *Phaseolus* spp. by *Agrobacterium*
542 *rhizogenes*, Nat. Protoc. 2 (2007) 1819-1824.
543 [39] A.P. Blackwell, T. Toussoun, W. Marasas, Fusarium species: an illustrated
544 manual for identification, Pennsylvania State University Press, University

ParkODonnell K (1992) Ribosomal DNA internal transcribed spacers are highly divergent in the phytopathogenic ascomycete Fusarium sambucinum (*Gibberella pulicaris*). Curr. Genet. 22 (1983): 213220.

[40] A.J. Able, D.I. Guest, M.W. Sutherland, Hydrogen peroxide yields during the incompatible interaction of tobacco suspension cells inoculated with *Phytophthora nicotianae*, Plant Physiol. 124 (2000) 899-910.

[41] A.L.W. Oller, E. Agostini, M.A. Talano, C. Capozucca, S.R. Milrad, H.A. Tigier, M.I. Medina, Overexpression of a basic peroxidase in transgenic tomato (*Lycopersicon esculentum* Mill. cv. *Pera*) hairy roots increases phytoremediation of phenol, Plant Sci. 169 (2005) 1102-1111.

[42] D. Cao, W. Hou, W. Liu, W. Yao, C. Wu, X. Liu, T. Han, Overexpression of *TaNHX2* enhances salt tolerance of ‘composite’ and whole transgenic soybean plants, Plant Cell Tiss. Org. 107 (2011) 541-552.

[43] E. L. Rech, G. R. Vianna, F. J. L. Aragao, High-efficiency transformation by biolistics of soybean, common bean and cotton transgenic plants. Nature Protocols 3 (2008) 410-418.

Figures Legends

Fig.1. Expression level of the *PvPOX1* gene induced by *F. oxysporum* f. sp. *phaseoli* isolate FOP-DM01 in common bean (*Phaseolus vulgaris* L.) genotype CAAS260205 (resistant) and BRB130 (susceptible). (A) Expression level of *PvPOX1* in roots ; (B) Expression level of *PvPOX1* in stems. *significant at $P \leqslant 0.05$, **significant at $P \leqslant 0.01$.

Fig. 2. Peroxidase activity in the roots and stems of common bean (*Phaseolus vulgaris* L.) genotype CAAS260205 (resistant) and BRB130 (susceptible) infected by *F. oxysporum* f. sp. *phaseoli* isolate FOP-DM01. (A) POX activity in roots; (B) POX activity in stems. *significant at $P \leqslant 0.05$, **significant at $P \leqslant 0.01$.

Fig. 3. H_2O_2 content in common bean (*Phaseolus vulgaris* L.) genotype CAAS260205 (resistant) and BRB130 (susceptible) infected by *F. oxysporum* f. sp. *phaseoli* isolate FOP-DM01. (A) H_2O_2 level in roots; (B) H_2O_2 level in stems. *significant at $P \leqslant 0.05$.

Fig.4. Disease assessment of common bean (*Phaseolus vulgaris* L.) susceptible genotype BRB130 infected with *F. oxysporum* at 14 d post inoculation (A) Phenotype of non-transformed (NT) and empty vector (EV) controls as well as *PvPOX1* hairy-root transgenic (PvPOX1) plants; (B) Disease scores of NT, EV and PvPOX1 plants based on 1 to 9 scale from Schoonhoven et al. (1987), where 1 = no visible disease symptoms; 3 = Very few wilted leaves combined with limited vascular discoloration of the root and hypocotyl; 5 = Approximately 25% of the leaves and branches exhibit wilting and chlorosis; 7 = Approximately 50% of the leaves and branches exhibit wilting, chlorosis, and limited necrosis with stunting of plants; 9 = Approximately 75% or more of the leaves and branches exhibit wilting, severe stunting, and necrosis with premature defoliation and plant death. *significant at $P \leqslant 0.05$.

592

593 **Fig.5.** Defense response in hairy roots of common bean (*Phaseolus vulgaris* L.)
594 susceptible genotype BRB130 non-transformed (NT) and empty vector (EV) controls
595 as well as *PvPOX1* hairy-root transgenic (PvPOX1) plants inoculated with *F.*
596 *oxysporum* f. sp. *phaseoli* isolate FOP-DM01. (A) *PvPOX1* gene expression degree;
597 (B) POX activity; (C) H_2O_2 level. *significant at $P \leqslant 0.05$, **significant at $P \leqslant 0.01$.
598

Fig. 1

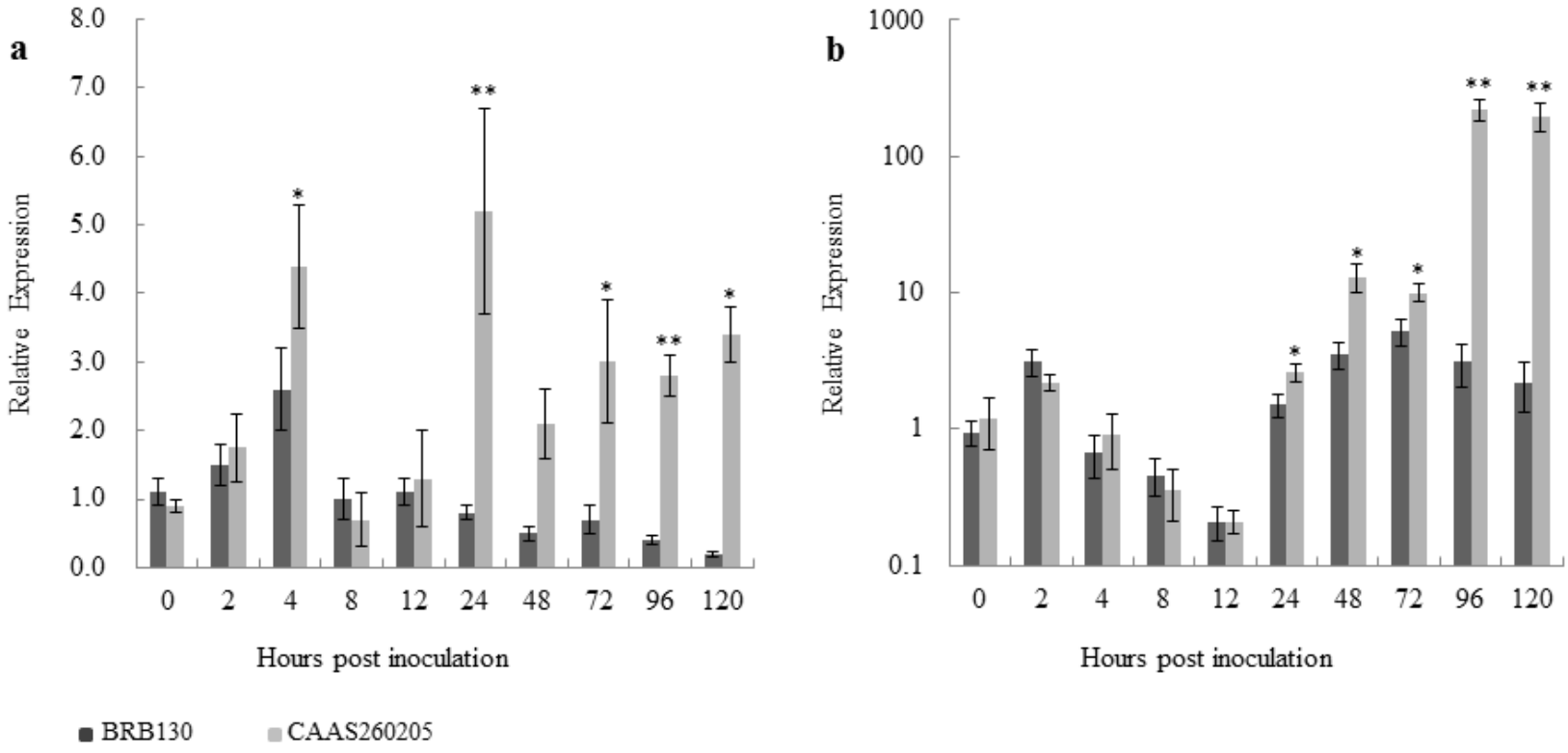

a
b
Relative Expression
Hours post inoculation
0
2
4
8
12
24
48
72
96
120
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
1000
100
10
1
0.1
*
**
BRB130
CAAS260205

Fig. 2

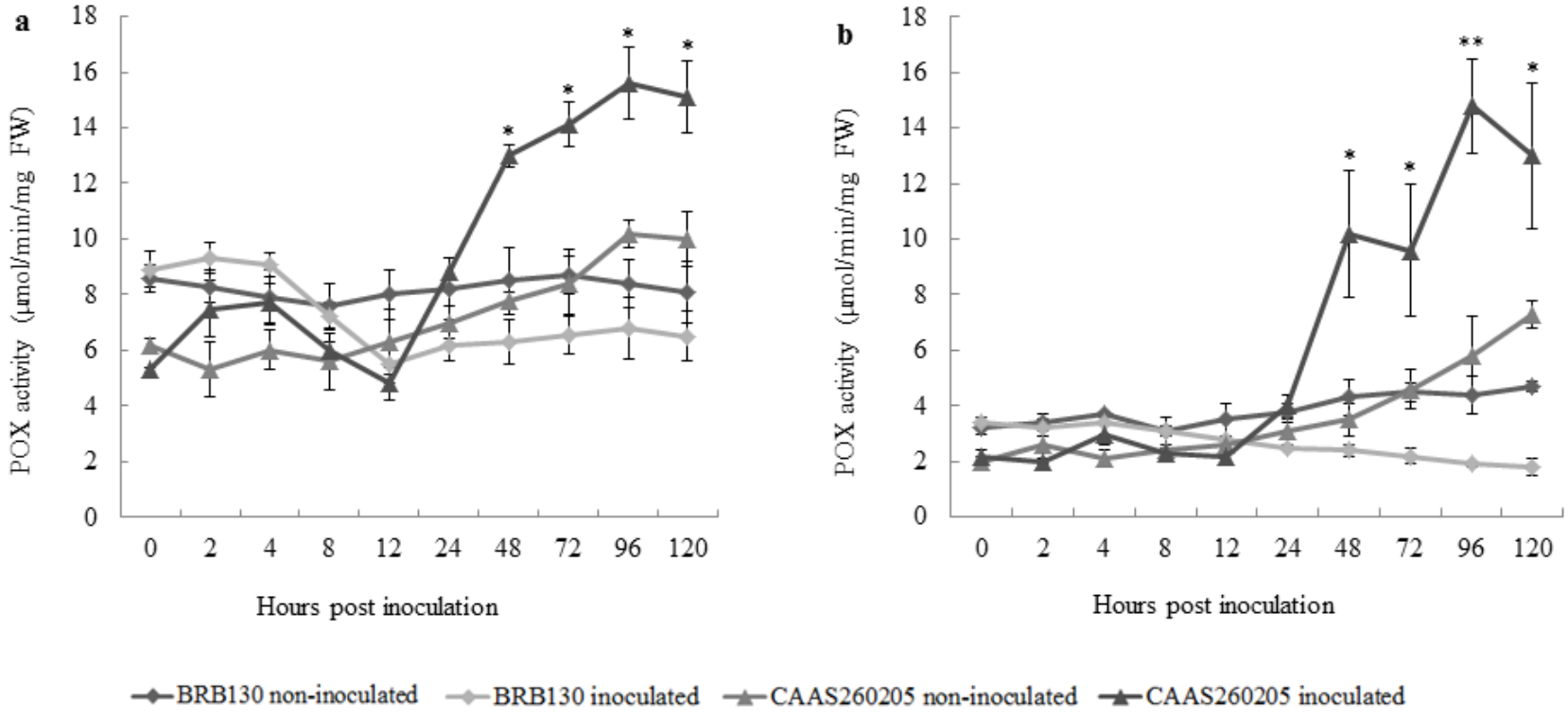
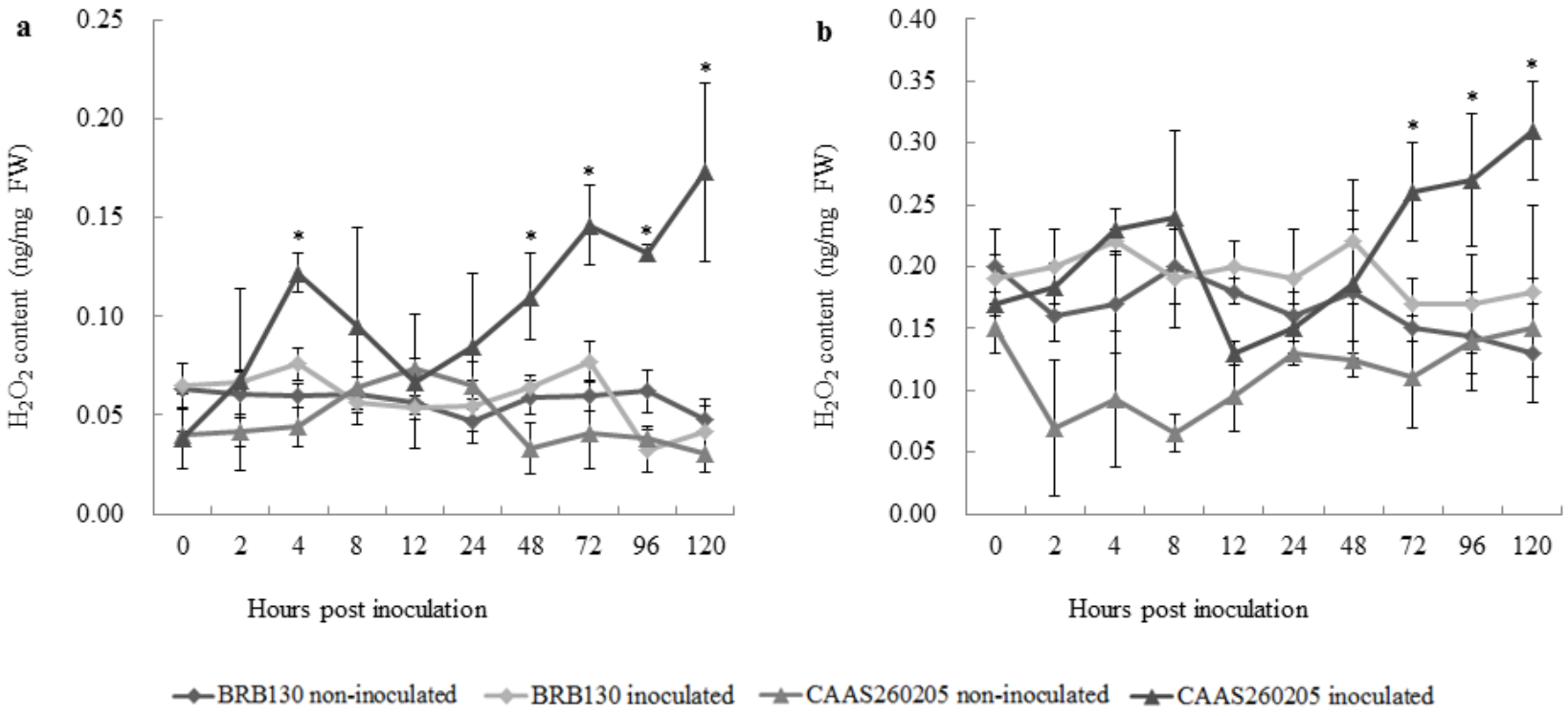
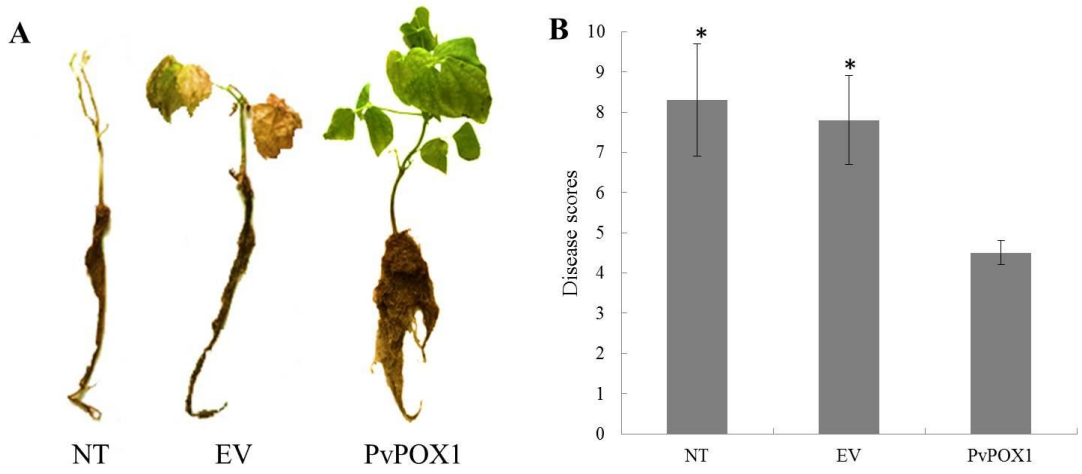
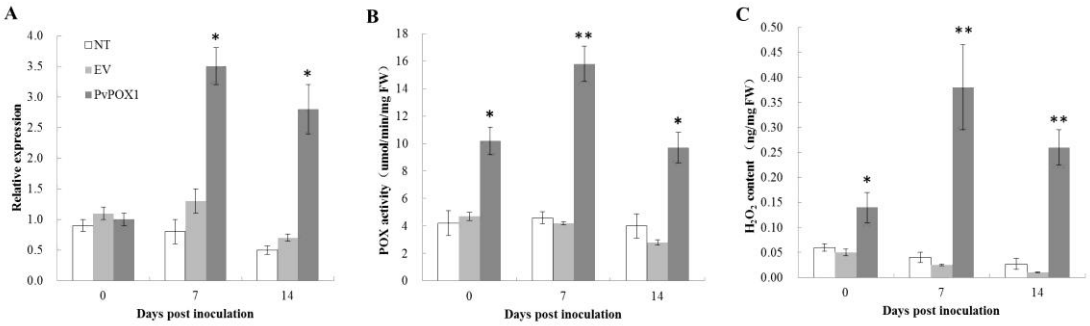

a
b
POX activity (μmol/min/mg FW)
Hours post inoculation
0
2
4
8
12
24
48
72
96
120
BRB130 non-inoculated
BRB130 inoculated
CAAS260205 non-inoculated
CAAS260205 inoculated

Fig. 3


a
b
H_2O_2 content (ng/mg FW)
0.25
0.20
0.15
0.10
0.05
0.00
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0 2 4 8 12 24 48 72 96 120
Hours post inoculation
BRB130 non-inoculated
BRB130 inoculated
CAAS260205 non-inoculated
CAAS260205 inoculated


A
NT
EV
PvPOX1
B
Disease scores
0
1
2
3
4
5
6
7
8
9
10
*
*
NT
EV
PvPOX1


A
NT
EV
PvPOX1
Relative expression
Days post inoculation
B
POX activity（umol/min/mg FW）
Days post inoculation
C
H_2O_2 content（ng/mg FW）
Days post inoculation